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[COMMUNICATION]

Effects of Puromycin and α-Amanitin on the Activity of Alkaline Phosphatase in Early Preimplantation Mouse Embryos

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ABSTRACT—The activity of nonspecific alkaline phosphatase (ALPase) was cytochemically and biochemically investigated in early preimplantation mouse embryos. The ALPase activity was cytochemically detected in embryos from 2- to 8-cell stage. The activity was exclusively localized on the adjacent cell membranes of the two blastomeres. This activity was biochemically first detected at the very low level in the 2-cell embryos. Biochemical assay revealed that in 4- to 8-cell embryos the ALPase activity increased dramatically. In these embryos, intense activity of ALPase was also detected cytochemically. When 2-cell embryos were treated with puromycin (15 μg/ml) in culture, the embryos could not develop to advanced stages and the expression of ALPase activity was inhibited. However, α-amanitin (2 and 10 μg/ml) suppressed the further cleavage, although it did not interfere with the expression of ALPase activity. The results suggest that mRNA molecules for ALPase of maternal origin exist in 2-cell embryos.

INTRODUCTION

Non-specific alkaline phosphatase (ALPase, E.C.3.1.3.1) is one of the well-known enzymes in early mammalian embryos. Cytochemical studies have shown the presence of its activity in 8-cell and more advanced mouse embryos [1–5]. Most of these investigators could not detect ALPase activity in fertilized and 2-cell embryos, except Mulnard and Huygens [4] who succeeded in detecting its activity in 2-cell embryos. They showed the precipitation of its reaction products in the adjacent cell membranes of the two blastomeres [4].

In the present study the expression of ALPase activity in the early mouse embryos was investigated in detail by employing both cytochemical and biochemical methods. Few information is available on the mechanism of ALPase expression except that concerning ascidian embryos, where ALPase has been employed as a histochemical marker for gut endodermal differentiation. This enzyme is considered to be maternal origin [6]. In the present investigation, inhibitors of transcription and translation were used to clarify whether the expression of ALPase activity in 2-cell embryos requires the *de novo* transcription and translation or not. The results suggest that the expression of ALPase activity requires *de novo* translation, but not transcription.

MATERIALS AND METHODS

Embryos

Female mice of strain 129/Sv were mated with male mice of the same strain after the superovulation by intraperitoneal injection of 5 i.u. pregnant mare serum gonadotropin (PMSG) followed by 5 i.u. human chorionic gonadotropin (hCG) 48 hr later. The 0 hr was defined as the time when hCG was injected. Two-cell stage and 4- to 8-cell stage embryos were flushed out from the oviduct 40–42 hr and 65–67 hr after hCG injection, respectively.

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Embryos were cultured in the standard egg culture medium [7]. The embryos of the experimental groups were cultured with medium containing either 15 μg/ml of puromycin or 2–10 μg/ml of α-amanitin for 3–24 hr.

Cytochemistry of alkaline phosphatase

Embryos were prefixed with ice-cold 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 20 min. They were then washed in the same buffer for more than 30 min. The lead citrate method [8] was used for the cytochemical demonstration of ALPase activity. The incubation was carried out at 37°C for 30–60 min in pH range of 9.2–9.3. No reaction was observed in control specimens which were incubated without substrate. After the incubation, specimens were washed with the buffer and embedded in agar blocks [9] and were fixed with ice-cold 1% OsO_4 solution for 60 min. They were dehydrated through a series of graded ethanols and embedded in epoxy resin.

For light microscopy, some of the specimens were washed with distilled water after the ALPase reaction and then dipped into diluted ammonium sulfide solution to detect the reaction products.

Biochemical assay of alkaline phosphatase

Fresh unfertilized eggs (>100), 2-cell embryos (>100) and 4- to 8-cell embryos (50–100) were collected for each assay. The blastomeres were broken by freezing and thawing. The ALPase activity was assayed by using Chung's method [10]. The reaction was carried out at 37°C for 4 hr. The amount of p-nitrophenol released from p-nitrophenol phosphate by the enzyme reaction was determined by spectrophotometry at the wave length of 410 nm. The specific activity of the enzyme was expressed in terms of nmol p-nitrophenol released in 1 hr per embryo. No enzyme activity was observed in the controls incubated in the same reaction mixture without the substrate.

RESULTS

Cytochemistry of alkaline phosphatase

ALPase activity was detected exclusively in the adjacent cell membranes of the two blastomeres in 2-cell embryos (Fig. 1a). Free cell surfaces of the blastomeres completely lacked ALPase activity. No ALPase activity was detected in the cell membranes of unfertilized eggs.

Biochemical assay of alkaline phosphatase activity

Embryos of 2-cell stage showed a weak ALPase activity (0.005 ± 0.007 nmol/hr/embryo, mean $\pm$ standard deviation, Table 1). In contrast, unfertilized eggs completely lacked its activity. In 4- to 8-cell embryos, ALPase activity drastically increased. The enzyme activities (0.15 ± 0.070 nmol/hr/embryo, Table 1) in 4- to 8-cell embryos were

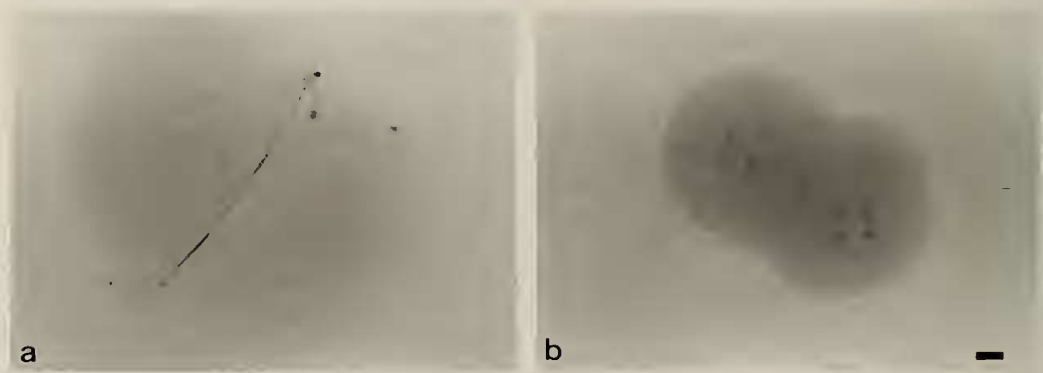

Fig. 1. Localization of ALPase activity in 2-cell mouse embryos and effects of puromycin on its activity. Mouse embryos were cultured with standard medium for 3 hr (a) and with medium containing 15 μg/ml of puromycin (b). Alkaline phosphatase activity is shown on the adjacent cell membranes of the two blastomeres in 2-cell embryos of control (a). However, the enzyme activity was not observed when 15 μg/ml of puromycin was added to the culture medium (b). Scale, 10 μm.

about 30 times higher than those in 2-cell embryos.

Effects of puromycin and α-amanitin on the development of 2-cell embryos in culture

When 2-cell embryos were cultured with standard medium for 24 hr, more than 50% embryos developed to advanced stages (Table 2). The effects of puromycin, an inhibitor of translation, on the development of 2-cell embryos were investigated. Puromycin added at a concentration of 15 μg/ml completely blocked the development of 2-cell embryos in culture. Also, the effects of α-amanitin, an inhibitor of transcription, on the development of 2-cell embryos were investigated. When α-amanitin was added to the medium at the dose of 10 μg/ml for 24 hr, most of embryos could not develop further (Table 2). However, more than 35% of the embryos could develop to advanced stages when 2-cell embryos were cultured with medium containing 2 μg/ml of α-amanitin (Table 2).

TABLE 1. Biochemical assays of alkaline phosphatase activity in preimplantation mouse embryos

Developmental stage	No. of experiments	No. of eggs or embryos	Alkaline phosphatase activity*
Unfertilized eggs	2	130–150	0±0
2-cell embryos (41–43 hr after hCG)	5	70–130	0.005±0.007
4- to 8-cell embryos (65 hr after hCG)	3	15– 35	0.150±0.070

* nmol p-nitrophenol released/hr/embryo (mean±standard deviation)

TABLE 2. Effects of puromycin and α-amanitin on the development of 2-cell embryos cultured for 24 hr

Group	Dose	No. of embryos	2-cell	3-cell	4-cell	5- to 8-cell
Control	—	125	52 (41.6%)	12 (9.6%)	50 (40%)	11 (8.8%)
Puromycin	15 μg/ml	64	64 (100%)	0 (0%)	0 (0%)	0 (0%)
α-amanitin	2 μg/ml	53	34 (64.1%)	3 (5.7%)	12 (22.6%)	4 (7.6%)
α-amanitin	10 μg/ml	60	56 (93.3%)	3 (5%)	1 (1.7%)	0 (0%)

TABLE 3. Effects of puromycin and α-amanitin on alkaline phosphatase activity in 2-cell embryos cultured for 24 hr

Group	Dose	No. of embryos	Alkaline phosphatase activity*		
			+	±	−
Control	—	15	12	2	1
Puromycin	15 μg/ml	9	0	4	5
α-amanitin	2 μg/ml	6	5	1	0
α-amanitin	10 μg/ml	22	10	7	5

* +: positive reaction
±: weakly positive reaction
−: negative reaction

Effects of puromycin and α-amanitin on alkaline phosphatase activity

Most of the 2-cell embryos cultured with normal medium for 24 hr expressed ALPase activity (Table 3). When 2-cell embryos were treated in culture with 15 μg/ml of puromycin for 24 hr, most of the embryos showed a very weak or no ALPase activity (Fig. 1b and Table 3). In contrast to puromycin, α-amanitin (2 and 10 μg/ml) did not inhibit the expression of ALPase activity (Table 3). Although 10 μg/ml of α-amanitin blocked the development of 2-cell embryos in culture, it did not suppress ALPase activity.

DISCUSSION

In the present study, ALPase activity was demonstrated in 2-cell mouse embryos both cytochemically and biochemically. Although ALPase activity was detected cytochemically in 2-cell mouse embryos by Mulnard and Huygens [4], their observations have not been supported by the biochemical analysis [5]. The present report, for the first time, describes the presence of ALPase activity in 2-cell embryos as demonstrated by biochemical means. The reason for the success in the detection of ALPase activity in 2-cell embryos probably due to the fact that the author used more than 100 fresh embryos for the assay.

When 2-cell embryos were treated with puromycin (15 μg/ml) for 24 hr, they could neither undergo further cleavage nor express ALPase activity. It is probable that the suppression of ALPase activity is due to the inhibition of *de novo* synthesis of ALPase molecules, since puromycin is known to inhibit translation. It has been known that puromycin inhibits in the concentration ranges of 4.7–94 μg/ml, the protein synthesis in rabbit reticulocyte [11]. When 2-cell embryos were treated with α-amanitin (2 or 10 μg/ml) for 24 hr, the development was inhibited, but the expression of ALPase activity was not suppressed. From the results it was proposed that the ALPase activity in 2-cell embryos was expressed without *de vovo* synthesis of mRNA, since α-amanitin is an inhibitor of RNA polymerase II. α-amanitin (0.4–40 μg/ml) is known to completely inhibit RNA polymerase II activity in calf thymocytes [12]. These results suggest that mRNA molecules for ALPase of maternal origin exists in 2-cell embryos. In contrast, the increase of ALPase activity in 4- to 8-cell embryos may be due to the embryonic transcription and translation, since ALPase activity increased drastically from 2-cell to 8-cell stage.

The 1- and 2-cell stages of development of a mouse embryo are thought to be dependent on the use of inherited maternal mRNA and on the operation of post-transcriptional regulators [13]. It is suggested that changes in the protein synthetic profile occur between the early 2-cell stage and the late 2-cell stage, and much of the maternally inherited mRNA is inactivated rapidly [14]. The stage of the 2-cell embryos used in the present study corresponds to the late 2-cell stage, since the embryos were collected from the oviduct 40-42 hr after hCG injection. Therefore, it is conceivable that the changes in the ALPase synthesis occur between the late 2-cell stage and the 4-cell stage. Although the difference between their results and the present findings cannot be fully explained, they might be attributed to difference in kind of proteins used. Molecular analyses of mRNA for ALPase in the mouse embryos at the late 2-cell stage remain to be investigated in a future study.

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